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Pin1 as a Biomarker of ER+ Breast Cancers to Predict the Response to Tamoxifen and mTOR Inhibitors

PRINCIPAL INVESTIGATOR:
Theresa Barberi

CONTRACTING ORGANIZATION:
Duke University
Durham, NC 27708

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Theresa Barberi

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14. ABSTRACT
Preliminary data from our lab showed that immortalized Pin1 null mouse embryo fibroblasts (MEFs) had decreased S6K phosphorylation and increased Akt phosphorylation. Since Akt and S6K activity have been shown to influence the sensitivity of cells to both Tamoxifen and mTOR inhibitors, we hypothesized that Pin1 could serve as a biomarker to help predict the response of estrogen receptor positive (ER+) breast tumors to these therapies. This preliminary data was not reproducible in primary Pin1 null MEFs. Although Pin1 binds phosphorylated S6K, we found no evidence to support a role for Pin1 in modulating S6K and Akt activity. We did, however, find a modest but reproducible defect in global protein synthesis and G0 exit. To further characterize the in vivo role of Pin1 in protein synthesis and proliferation, we turned our attention to cells of the immune system. Upon LPS challenge, we find a marked defect in the ability of Pin1 null mice to produce many cytokines, including IL-6. This finding has been confirmed in primary Pin1 null MEFs, which also exhibit a defect in LPS-stimulated IL-6 secretion. Future studies will seek to identify the mechanism by which Pin1 regulates IL-6 production. This information will then be applied to breast cancer cells, as IL-6 has been reported to play a role in breast cancer progression and influence the sensitivity of breast cancer cells to chemotherapeutic agents.

15. SUBJECT TERMS
Pin1, S6 Kinase, Akt, Rapamycin, Tamoxifen, LPS, IL-6, Inflammation

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INTRODUCTION

Five years of Tamoxifen therapy is the standard treatment for patients harboring breast tumors that express the estrogen receptor (ER). Not all patients respond to Tamoxifen, however, and 1/3 of those patients that initially respond eventually develop resistance. It has been reported that Akt activity may predict the response of ER+ tumors to Tamoxifen. Additionally, inhibitors of mTOR, a downstream effector of Akt, are being investigated for their ability to combat Tamoxifen resistant tumors. Cells with high Akt and S6K activity have been shown to exhibit increased sensitivity to the mTOR inhibitor Rapamycin. Preliminary data from our lab suggested that the prolyl isomerase Pin1 could modulate the activity and phosphorylation of Akt and S6K. Specifically, we found that S6K phosphorylation was decreased and Akt phosphorylation was increased in immortalized Pin1 null mouse embryo fibroblasts (MEFs). This led us to hypothesize that Pin1 could serve as a biomarker to predict the response of ER+ breast tumors to Tamoxifen therapy and mTOR inhibitors. Experiments conducted in primary MEFs were unable to confirm our original findings; we found no evidence to support a role for Pin1 in modulating S6K phosphorylation or Akt phosphorylation. We did, however, find that the absence of Pin1 in MEFs led to a small but reproducible defect in global protein synthesis and G0 exit. The immune system is comprised of multiple cell types that, when activated, must rapidly synthesize new protein and/or proliferate in order to mount a successful immune response. We looked to the immune system to further characterize the in vivo role of Pin1 in protein synthesis and proliferation. Upon lipopolysaccharide (LPS) challenge, Pin1 null mice exhibit a marked inability to produce many different cytokines, including IL-6. We have confirmed this finding in Pin1 null primary MEFs, which also exhibit a defect in IL-6 secretion in response to LPS. Serum IL-6 levels have been correlated with patient survival in ER- metastatic breast cancer, and blocking IL-6 secretion in Ras transformed cells hampers their ability to form tumors in mice. There is also evidence from breast cancer cell lines that suggests IL-6 production may confer resistance to chemotherapeutic agents such as doxorubicin. For these reasons, we are interested in identifying a role for Pin1 in modulating cytokine production and inflammation, and would like to then apply these findings to suggest a role for Pin1 in cytokine-dependent survival of breast cancer cells.

BODY

Updates for the Original Statement of Work:

In the months following submission of the grant application, much work was done to confirm our preliminary results. The data presented in the proposal showed alterations in S6K and Akt phosphorylation. Some of this data was generated using MEFs that had been immortalized by stably expressing a dominant negative version of p53, allowing the cells to be passaged many times in culture. When we tried to confirm our results in primary MEFs, we were unable to detect all of the defects initially identified in the immortalized Pin1 null MEFs.

Using primary wild type (WT) MEFs, we were able to confirm that GST-Pin1 binds S6K when it is phosphorylated on T389 (**Figure 2**). In the absence of Pin1, however, we did not detect a defect in the phosphorylation of S6K (**Figure 3A, 3B**) or in the ability of the mTOR inhibitor Rapamycin to inhibit S6K phosphorylation (**Figure 3A**). mTOR can form two distinct complexes in the cell (**Figure 1**). The complex upstream of S6K is TORC1, which includes mTOR and Raptor. The second complex, TORC2, includes mTOR and Rictor. mTOR is thought to exist in an equilibrium between these two complexes[1, 2]. To determine if this equilibrium is shifted in the absence of Pin1, we assessed the ability of mTOR to form a complex with Rictor by immunoprecipitating Rictor and quantifying the amount of associated mTOR. We did not find any defects in the ability of mTOR to bind Rictor in the absence of Pin1

(**Figure 4**). Since cells lacking S6K have been found to be smaller in size than their wild type counterparts, we measured the area of our primary Pin1 null MEFs[3]. We found that the absence of Pin1 did not alter the area of primary MEFs (**Figure 5**). S6K activity has also been shown to influence the sensitivity of cells to Rapamycin; cells with high S6K activity are more easily inhibited and those with low S6K activity are less sensitive to the drug[4]. We measured Rapamycin sensitivity in our Pin1 null MEFs by proliferation assay, and found no differences in sensitivity compared to wild type MEFs (**Figure 6**).

Our data from primary MEFs did not support a role for Pin1 in modulating S6K phosphorylation and activity, or in altering cell sensitivity to the mTOR inhibitor Rapamycin. As a result, similar experiments were not carried out in breast epithelial cells or in breast cancer cell lines, as described in ***Original Task 1***.

Data and Rationale Supporting the Revised Statement of Work:

Revised Task 1:

Our lab (unpublished data) and others have shown that primary Pin1 null MEFs exhibit a delay in their ability to re-enter the cell cycle from quiescence[5]. Because protein synthesis is important for G0 exit, and because we were initially interested in the mTOR-S6K pathway controlling protein synthesis, we measured protein synthesis in wild type and Pin1 null primary MEFs and found a small but reproducible defect in protein synthesis in MEFs lacking Pin1 (**Figure 7**). Since MEFs are not a physiologically relevant cell type in the adult mouse, we sought to find a better model for studying this defect. We turned our attention to the immune system because it is comprised of several cell types that, when activated, must rapidly synthesize new protein and/or proliferate in order to achieve an effective immune response. In a pilot experiment, we challenged the immune system of 3 WT and 3 Pin1 null mice by injecting the bacterial cell wall component LPS into the peritoneal cavity. PBS was injected into 2 WT mice, and 2 KO mice to serve as a negative control. Seventeen hours later, the mice were sacrificed and serum was collected. A fraction of the serum from each animal was pooled into one of four groups (WT PBS, WT LPS, KO PBS, or KO LPS) and screened for markers of inflammation, which include cytokines, growth factors, and other inducible proteins. The results of this screen revealed that many of these factors were greatly reduced in the KO LPS animals compared to the WT LPS animals (**Table 1**). We used ELISA to confirm these results for one cytokine, IL-6, and found IL-6 levels to be much lower in the Pin1 null mice treated with LPS than in the WT mice treated with LPS (**Figure 8**).

Upon LPS challenge, mature dendritic cells (mDC) should accumulate in the spleen. mDC arise from the activation of immature DC (iDC) that normally reside in the spleen, and also from the recruitment of circulating monocytes into the spleen, where they undergo differentiation into DC[6]. To determine whether mDC were able to accumulate in the spleens of our mice, splenocytes from each animal were stained for markers of mDCs seventeen hours after LPS administration. We found that the WT mice treated with LPS accumulated mDCs in their spleens as expected, but that the LPS-treated Pin1 null animals had a severe defect in this accumulation (**Figure 9**).

Since we inject LPS intraperitoneally, inflammation is first initiated by cells that reside in the peritoneal cavity. Two abundant cell types present in the peritoneal cavity are macrophages and neutrophils, both of which can respond to LPS. To be sure the mDC defect was not due to the absence of such cells in Pin1 null mice we used flow cytometry to reveal the presence of macrophages and neutrophils (**Figure 10A**). Additionally some peritoneal cells were placed in culture overnight. The next day, adherent cells were removed, stained for macrophage markers, and analyzed by flow cytometry. This result further confirmed macrophages to be present in WT and Pin1 null mice (**Figure 10B**).

An important part of amplifying and maintaining inflammation is the mobilization, recruitment, and activation of cells in other tissues that are not a part of the local response. This process is reliant on the proper functioning of endothelial cells, which are capable of responding to both LPS and cytokines. Endothelial cells can be activated directly by LPS, or they can be activated indirectly by cytokines produced by other cell types. Upon activation, endothelial cells themselves will produce cytokines and chemokines, which are important for the recruitment and migration of immune cells to the site of inflammation and to secondary lymphoid organs[9]. The defect we see in mDC accumulation in the spleen in our Pin1 null mice could be the result of defective DC maturation, and/or the result of a defect in monocyte recruitment and migration. To determine whether the defect in mDC accumulation in Pin1 null mice is cell intrinsic, we will generate iDC *in vitro* by differentiating bone marrow using GM-CSF and IL-4. We will then evaluate the ability of bone marrow-derived iDC (BMDC) to mature and produce cytokines by staining BMDC for maturation markers, and measuring cytokine accumulation in the media by ELISA. If data show that Pin1 null BMDC can mature and secrete cytokines normally *in vitro*, it would support the idea that their inability to accumulate in the spleen is due to migration defects. This idea will then be further explored by performing *in vitro* and *in vivo* assays to directly measure the ability of DC to migrate. Additionally, the role of endothelial cells in this process can be investigated by purifying endothelial cells from the mouse, and then measuring the ability of these cells to produce cytokines and chemokines in response to LPS and other cytokines.

In our model of LPS stimulation, cells of the innate immune system initiate inflammation. Signals emanating from these cells should then activate members of the adaptive immune system, which is composed of B and T lymphocytes. In our initial screen, Pin1 null mice treated with LPS exhibited a decrease in the levels of many cytokines that are produced by activated lymphocytes, suggesting the activation of these cells may be defective. To determine if there exist defects in lymphocyte activation in our Pin1 null mice, we plan to administer LPS for seventeen hours, and then remove the spleens. Whole splenocytes will be stained for markers of activated T cells and B cells. Additionally, RNA can be harvested from splenocytes and analyzed by q-rtPCR for cytokine expression, which will further inform us of whether activation signals have reached the spleen.

Revised Task 2:

Male and female Pin1 null mice exhibit markedly reduced fertility due to defects in the proliferation of primordial germ cells. This defect renders them incapable of producing litters[10]. Animals that are heterozygous at the Pin1 locus also display reduced fertility, but are capable of producing litters, albeit to a lesser extent than wild type animals. Because the generation of Pin1 null animals is limiting, we decided to investigate LPS signaling in primary MEFs, which express toll-like receptor 4 (TLR4), the receptor that binds LPS and initiates an intracellular signaling cascade leading to cytokine production[11].

When we stimulated primary MEFs *in vitro* with LPS, we saw a defect in the ability of Pin1 null MEFs to secrete IL-6 into the media, but not TNFα (**Figure 13A**). We also harvested RNA from these cells, and found that IL-6 mRNA is decreased in Pin1 null MEFs, but not TNFα (**Figure 13B**). Because Pin1 has been shown to regulate the mRNA stability of GM-CSF mRNA in both eosinophils and T cells[12, 13], we examined GM-CSF mRNA accumulation in our MEFs as well. Upon LPS stimulation, GM-CSF mRNA was induced to the same extent in both WT and Pin1 null MEFs, indicating that Pin1 does not regulate GM-CSF mRNA stability in this cell type (**Figure 13B**).

Because there is precedent for Pin1 to modulate mRNA stability, we plan to measure the stability of IL-6 mRNA in our Pin1 null MEFs by inhibiting *de novo* transcription using Actinomycin D. Cells will then be stimulated with LPS and mRNA will be measured at various times via q-rtPCR. If we see a defect in IL-6 mRNA stability in Pin1 null MEFs, we will assess whether there is a defect in the association of RNA-binding proteins with IL-6 mRNA, as this

was demonstrated to be the case for GM-CSF mRNA in eosinophils treated with the Pin1 inhibitor Juglone[12]. If the absence of Pin1 does not affect IL-6 mRNA stability, then it is likely that the there is a defect in LPS-stimulated IL-6 transcription.

LPS signals through Toll-like Receptor 4 (TLR4) to activate multiple pathways that induce the transcription of many inflammatory cytokines[14-16]. We will assess whether TLR4 signaling pathways are intact in our Pin1 null MEFs by evaluating the phosphorylation of ERK1/2, JNK, and p38 MAPK in response to LPS by immunoblot[16]. Transcription factors identified downstream of these pathways include NF-κB, NF-IL6, IRF3, IRF5, Stat5, and AP-1[14, 15, 17]. Regulation of cytokine production is complex, however, as many of these transcription factors bind the same promoters and cooperatively regulate the transcription of target genes[15, 16, 18]. In the case of IL-6, it has been reported that NF-κB, NF-IL6, AP-1, Stat3 and Stat5 are able to transcriptionally regulate its production[15, 17, 19, 20]. To aid in narrowing down which transcription factor(s) may have altered activity in our Pin1 null MEFs, we will evaluate the production of other cytokines and genes whose transcription has been reported to be regulated by these transcription factors. q-rtPCR will be utilized to measure the production of IL-1 and IL-12 (NF-κB targets), IFNβ (IRF3 target), Bcl-x and MIP-1α (Stat5 targets) in MEFs stimulated with LPS. Additionally, it has been reported that inhibition of Jak2, the kinase upstream of Stat5, specifically influences IL-6 transcription, but not TNFα or GM-CSF[17]. Since we see a similar phenotype in our Pin1 null MEFs, we will determine if Jak2-Stat5 signaling is perturbed. Phosphorylation of Jak2 and Stat5 can be measured by immunoblot in response to LPS. Additionally, SOCS-1 is a negative regulator of Jak2, and has been previously shown to modulate the ubiquitination and turnover of the Pin1 target NF-κB p65[21]. To determine if Pin1 similarly modulates Jak2 regulation by SOCS-1, we will perform immunoprecipitation experiments to look at the association of SOCS-1 with Jak2 in WT and Pin1 null MEFS. To verify candidate transcription factors whose activity may be altered in the absence of Pin1, luciferase reporter constructs for these transcription factors will be expressed in MEFs so that upon stimulation and promoter activation, luciferase is produced and can be quantified.

Revised Task 3:

In recent years, it has become increasingly clear that there is a link between the immune system and cancer. Innate and adaptive immune cells have been shown to play various roles in the initiation and maintenance of tumors[22, 23]. Additionally, tumor cells are able to produce cytokines, chemokines, and growth factors that influence many immune system processes in addition to providing autocrine or paracrine signals that stimulate tumor cell proliferation and survival[24-26]. IL-6 is a cytokine that is implicated in tumor growth and metastasis. Many types of cancer are associated with elevated circulating IL-6 levels, including multiple myeloma, and cancers of the colon, prostate, ovary, and breast[27, 28]. Furthermore, serum IL-6 levels are negatively correlated with survival in receptor negative breast cancers[27, 29]. Tumor-derived IL-6 may also play a role in resistance to chemotherapeutic agents. It has been shown that pre-treatment of the MCF-7 breast cancer cell line with IL-6 leads to enhanced resistance to the chemotherapeutic agent doxorubicin. Additionally, multidrug resistant MCF-7 cells produce elevated levels of IL-6 protein[30]. For these reasons, we are interested in evaluating if Pin1 is able to modulate IL-6 production in breast cancer cells.

Future experiments will determine if there is a correlation between Pin1 mRNA expression and IL-6 production in various breast cancer cell lines. It has been shown that the estrogen receptor (ER) negative breast cancer cell lines MCF-7, T47D, and BT474 do not secrete detectable IL-6 protein in culture. ER positive cell lines, such as MD-MB-231 and MD-MB-468, do produce detectable IL-6 levels in culture[31]. We plan to measure Pin1 mRNA and protein in these cell lines and correlate it to IL-6 production. Additionally, we will manipulate Pin1 expression in these cell lines by either over-expressing Pin1 or knocking down its expression using RNAi. We will use the same approaches as those described for MEFs in *Revised Task 2* in

order to determine if the mechanism of IL-6 regulation is conserved between MEFs and breast cancer cells lines. We will then determine how these alterations in Pin1 expression influence breast cancer cell production of IL-6 and sensitivity to chemotherapeutic agents and Tamoxifen.

KEY RESEARCH ACCOMPLISHMENTS

- Pin1 modulates the production of many inflammatory cytokines, including IL-6, in response to LPS in mice.
- Pin1 regulates the accumulation of mDCs in the spleens of mice challenged with LPS.
- Pin1 modulates IL-6 mRNA accumulation in response to LPS in MEFs.

REPORTABLE OUTCOMES

Pin1 Regulates the Inflammatory Response. Barberi TJ, Racioppi L, and Means AR. Poster presented at Duke University Medical Center Department of Pharmacology and Cancer Biology Annual Retreat, Wrightsville Beach, NC (September 26-28, 2008).

Pin1 Regulates the Inflammatory Response. Barberi TJ, Racioppi L, and Means AR. Poster presented at Duke University Medical Center Annual Biological Sciences Graduate Student Symposium, Durham, NC (November 14, 2008).

CONCLUSION

We have shown that Pin1 null mice challenged with LPS have a defect in their ability to mount an inflammatory response. Additionally, MEFs lacking Pin1 do not accumulate IL-6 mRNA to the same extent as WT MEFs when stimulated with LPS *in vitro*. Collectively, our data support a role for Pin1 in modulating the cellular production of IL-6 and the process of inflammation in the mouse.

There is a growing body of evidence suggesting that IL-6 is an important prognostic factor for breast cancer progression. In patients with ER- metastatic breast cancer, serum IL-6 levels have been shown to correlate with patient survival. Additionally, IL-6 secretion enhances the ability of transformed cells to form tumors in mice and confers resistance to chemotherapeutic agents in breast cancer cell lines. Future studies are aimed at identifying the mechanism by which Pin1 modulates IL-6 production and the role of Pin1 in the response of breast cancer cells to chemotherapeutic agents. As a regulator of IL-6 production, we believe that Pin1 may be a useful therapeutic target for preventing breast tumor progression and resistance to chemotherapeutic agents.

REFERENCES

1. Chiang, G. G. and Abraham, R. T. Targeting the mTOR signaling network in cancer. Trends Mol Med, *13:* 433-442, 2007.
2. Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. Rictor, a novel binding partner of mTOR,

defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol, *14:* 1296-1302, 2004.
3. Ruvinsky, I. and Meyuhas, O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci, *31:* 342-348, 2006.
4. Noh, W. C., Mondesire, W. H., Peng, J., Jian, W., Zhang, H., Dong, J., Mills, G. B., Hung, M. C., and Meric-Bernstam, F. Determinants of rapamycin sensitivity in breast cancer cells. Clin Cancer Res, *10:* 1013-1023, 2004.
5. Fujimori, F., Takahashi, K., Uchida, C., and Uchida, T. Mice lacking Pin1 develop normally, but are defective in entering cell cycle from G(0) arrest. Biochem Biophys Res Commun, *265:* 658-663, 1999.
6. Serbina, N. V., Jia, T., Hohl, T. M., and Pamer, E. G. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol, *26:* 421-452, 2008.
7. Nathan, C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol, *6:* 173-182, 2006.
8. De Filippo, K., Henderson, R. B., Laschinger, M., and Hogg, N. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. J Immunol, *180:* 4308-4315, 2008.
9. Dauphinee, S. M. and Karsan, A. Lipopolysaccharide signaling in endothelial cells. Lab Invest, *86:* 9-22, 2006.
10. Atchison, F. W., Capel, B., and Means, A. R. Pin1 regulates the timing of mammalian primordial germ cell proliferation. Development, *130:* 3579-3586, 2003.
11. Kurt-Jones, E. A., Sandor, F., Ortiz, Y., Bowen, G. N., Counter, S. L., Wang, T. C., and Finberg, R. W. Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity. J Endotoxin Res, *10:* 419-424, 2004.
12. Shen, Z. J., Esnault, S., and Malter, J. S. The peptidyl-prolyl isomerase Pin1 regulates the stability of granulocyte-macrophage colony-stimulating factor mRNA in activated eosinophils. Nat Immunol, *6:* 1280-1287, 2005.
13. Esnault, S., Shen, Z. J., Whitesel, E., and Malter, J. S. The peptidyl-prolyl isomerase Pin1 regulates granulocyte-macrophage colony-stimulating factor mRNA stability in T lymphocytes. J Immunol, *177:* 6999-7006, 2006.
14. Doyle, S. L. and O'Neill, L. A. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. Biochem Pharmacol, *72:* 1102-1113, 2006.
15. Kawai, T. and Akira, S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med, *13:* 460-469, 2007.
16. Guha, M. and Mackman, N. LPS induction of gene expression in human monocytes. Cell Signal, *13:* 85-94, 2001.
17. Kimura, A., Naka, T., Muta, T., Takeuchi, O., Akira, S., Kawase, I., and Kishimoto, T. Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK-STAT. Proc Natl Acad Sci U S A, *102:* 17089-17094, 2005.
18. Lu, Y. C., Yeh, W. C., and Ohashi, P. S. LPS/TLR4 signal transduction pathway. Cytokine, *42:* 145-151, 2008.
19. Xiao, W., Hodge, D. R., Wang, L., Yang, X., Zhang, X., and Farrar, W. L. NF-kappaB activates IL-6 expression through cooperation with c-Jun and IL6-AP1 site, but is independent of its IL6-NFkappaB regulatory site in autocrine human multiple myeloma cells. Cancer Biol Ther, *3:* 1007-1017, 2004.
20. Samavati, L., Rastogi, R., Du, W., Huttemann, M., Fite, A., and Franchi, L. STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol Immunol, 2009.
21. Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y. C., Wulf, G., Rottapel, R., Yamaoka, S., and Lu, K. P. Regulation of NF-kappaB signaling by Pin1-dependent prolyl

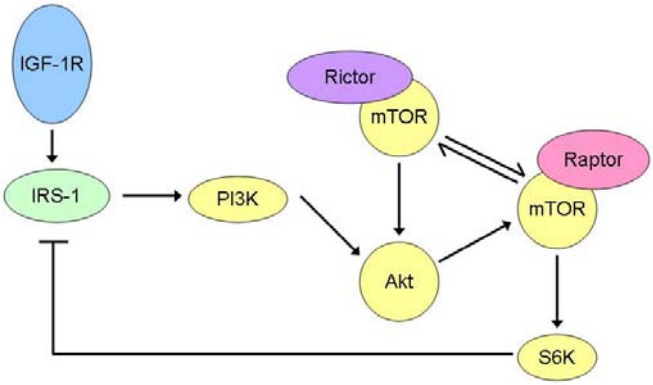
isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol Cell, *12:* 1413-1426, 2003.
22. Marigo, I., Dolcetti, L., Serafini, P., Zanovello, P., and Bronte, V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev, *222:* 162-179, 2008.
23. Ostrand-Rosenberg, S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev, *18:* 11-18, 2008.
24. Ancrile, B. B., O'Hayer, K. M., and Counter, C. M. Oncogenic ras-induced expression of cytokines: a new target of anti-cancer therapeutics. Mol Interv, *8:* 22-27, 2008.
25. Nicolini, A. and Carpi, A. Immune manipulation of advanced breast cancer: an interpretative model of the relationship between immune system and tumor cell biology. Med Res Rev, *29:* 436-471, 2009.
26. Nicolini, A., Carpi, A., and Rossi, G. Cytokines in breast cancer. Cytokine Growth Factor Rev, *17:* 325-337, 2006.
27. Mumm, J. B. and Oft, M. Cytokine-based transformation of immune surveillance into tumor-promoting inflammation. Oncogene, *27:* 5913-5919, 2008.
28. Naugler, W. E. and Karin, M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med, *14:* 109-119, 2008.
29. Bachelot, T., Ray-Coquard, I., Menetrier-Caux, C., Rastkha, M., Duc, A., and Blay, J. Y. Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. Br J Cancer, *88:* 1721-1726, 2003.
30. Conze, D., Weiss, L., Regen, P. S., Bhushan, A., Weaver, D., Johnson, P., and Rincon, M. Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. Cancer Res, *61:* 8851-8858, 2001.
31. Sasser, A. K., Sullivan, N. J., Studebaker, A. W., Hendey, L. F., Axel, A. E., and Hall, B. M. Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer. Faseb J, *21:* 3763-3770, 2007.

APPENDICES

None.

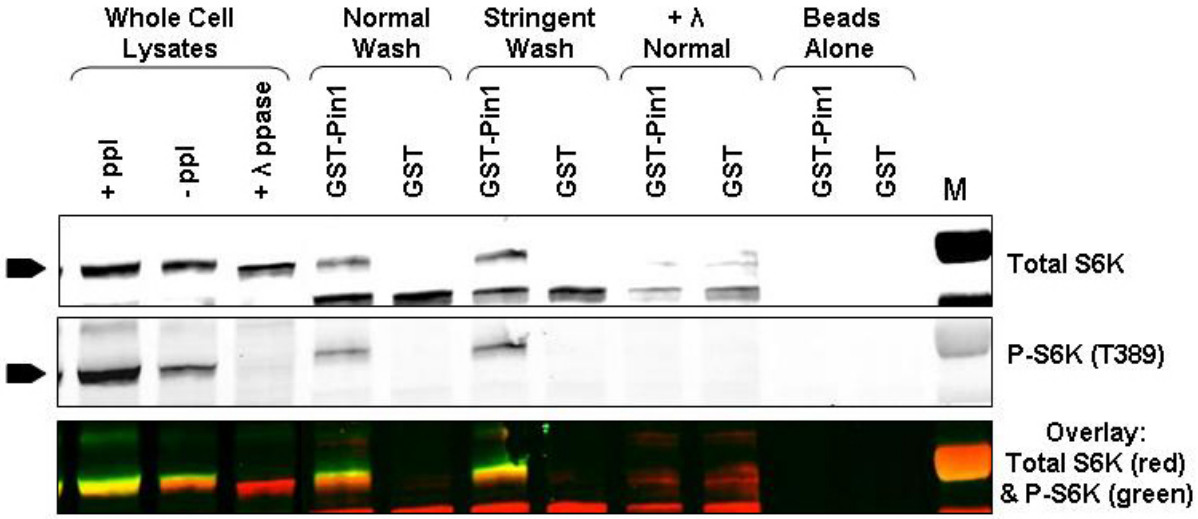
SUPPORTING DATA

Figure 1. The Akt-mTOR-S6K Signalling Pathway.



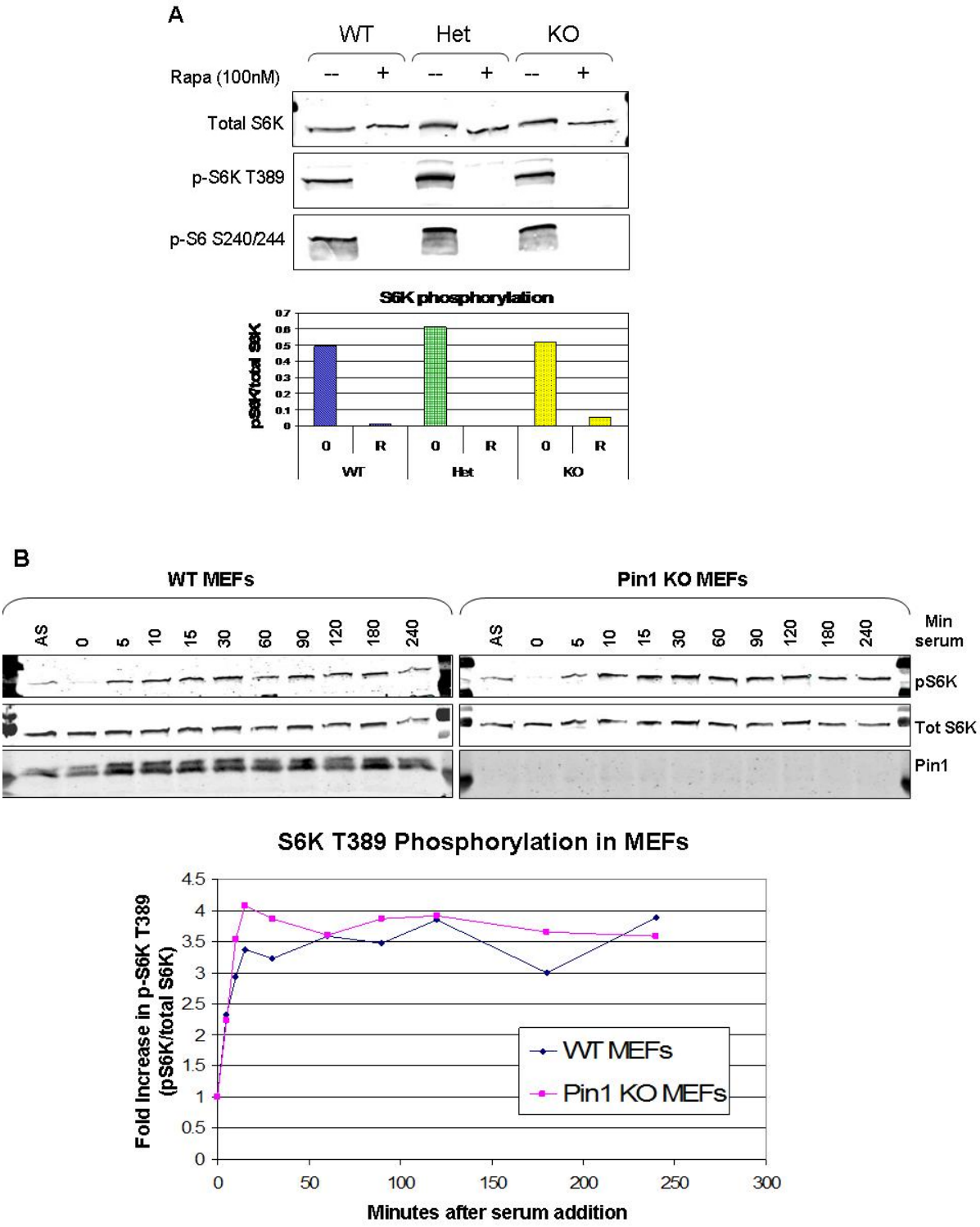
Mitogenic stimuli bind and activate their receptors on the cell surface (in this example, IGF-1R), and signal through IRS-1 to sequentially activate PI3K, Akt, mTOR, and S6K. Activated S6K is capable of phosphorylating IRS-1 on sites that promote its degradation, thereby creating a negative feedback loop.

Figure 2. GST-Pin1 binds S6K that is phosphorylated on T389.



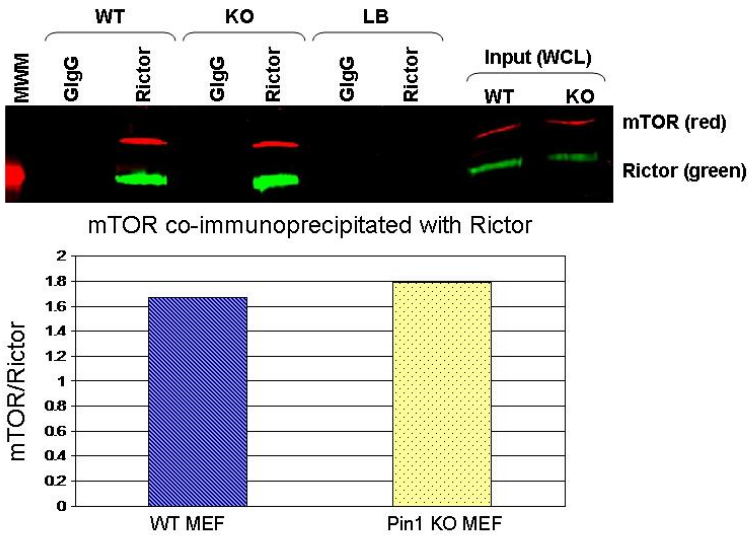
Primary wild type MEFs were either lysed in buffer containing phosphatase inhibitors (+ppI) or in buffer without phosphatase inhibitors (-ppI). Lambda phosphatase was added to the lysate that did not include phosphatase inhibitors to dephosphorylate proteins in the lysate (+λ ppase). A GST-Pin1 pull down was carried out by adding GSH-Agarose beads conjugated to GST-Pin1 or GST alone to both the lysate with phosphatase inhibitors (+ppI) and the lysate treated with lambda phosphatase (+λ ppase). The lysate containing phosphatase inhibitors (+ppI) was split, and one half was washed 4 times with lysis buffer by gentle inversion (Normal), and the other half was washed 4 times in RIPA buffer and vortexed vigorously between each wash (Stringent). The lysate containing lambda phosphatase was subjected to the Normal wash only. After washing, bound proteins were eluted from beads by the addition of SDS-Page sample buffer followed by boiling, and then analyzed by western blot. As a negative control, SDS-Page sample buffer was also added directly to GSH-Agarose beads conjugated to GST-Pin1 or GST, boiled, and also analyzed by western blot.

Figure 3. Primary Pin1 null MEFs do not exibit a defect in S6K phosphorylation.



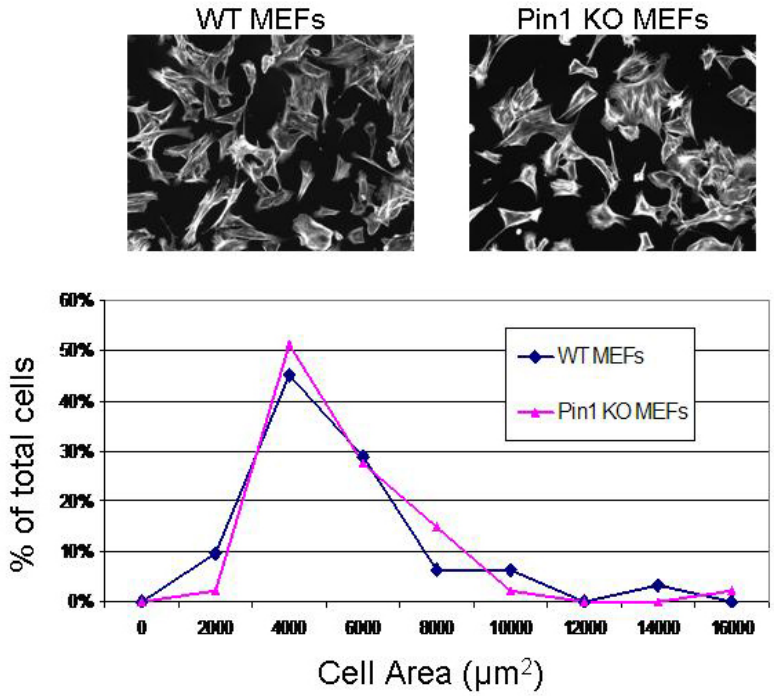
A) Asynchronous primary Pin1 +/+ (WT), Pin1 +/- (Het), and Pin1 -/- (KO) MEFs were treated with vehicle (0) or 100nM Rapamycin (R) for 60 minutes, lysed, and analyzed by western blot. **B)** Primary WT and Pin1 KO MEFs were starved of serum overnight and then re-stimulated with 10% heat-inactivated FBS for 0-240 minutes. Cells were then lysed and analyzed by western blot. (AS = Asynchronous control cells maintained in 10% heat-inactivated FBS).

Figure 4. The ability of mTOR to associate with Rictor is unaffected by the absence of Pin1.



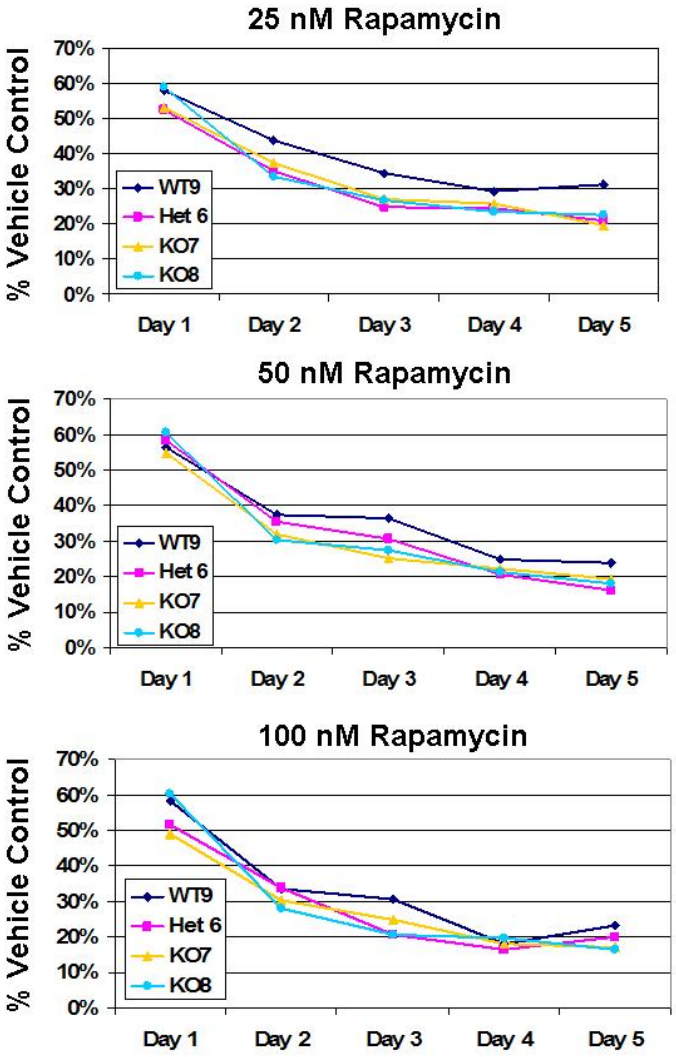
Rictor was immunoprecipitated in lysates from wild type (WT) and Pin1 null (KO) primary MEFs. Goat IgG (GIgG) served as a negative control for the Rictor immunoprecipitation. Lysis buffer alone (LB) served as another control and was subjected to immunoprecipitation with GIgG or Rictor. Immunoprecipitates were analyzed by western blot for Rictor (green) and mTOR (red). Band densities were quantified and the amount of bound mTOR per Rictor is plotted on the graph below. This is representative of three separate experiments.

Figure 5. The absence of Pin1 does not alter cell area in MEFs.



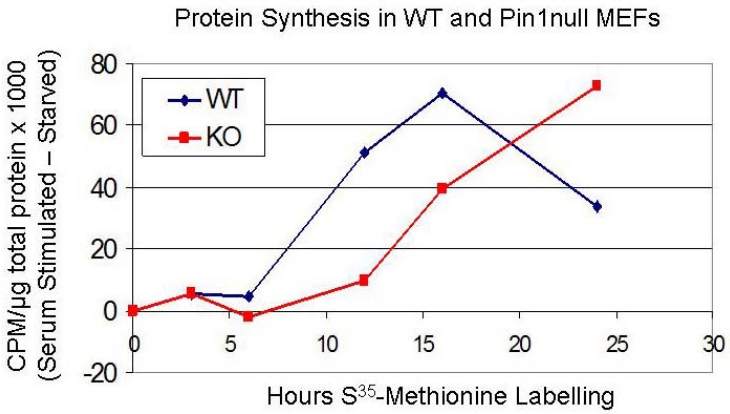
Asynchronous WT and Pin1 null MEFs were fixed, permeabilized, and stained with Rhodamine-Phalloidin, which binds F-actin, and allows easy detection of the cell periphery. Zeiss Axiovert software was used to manually trace the edges of each cell, and the cell area was then calculated.

Figure 6. Rapamycin inhibits the growth of WT and Pin1 null MEFs to similar extents.



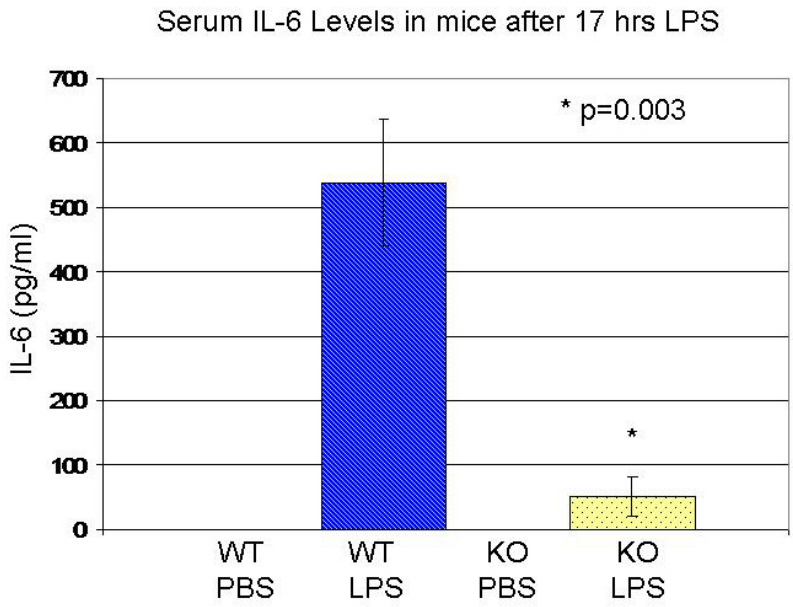
Primary MEFs from four embryos (WT9, Het6, KO7, KO8) were seeded into 96-well plates at 2000 cells/well. Cells were treated either with vehicle alone or with 25, 50, or 100 nM Rapamycin and allowed to grow for 1-5 days. Cell numbers were quantified each day by CyQuant assay and cell numbers in rapamycin treated wells were plotted as a percentage of cells present in wells that received vehicle alone. (WT = Pin1 +/+; Het = Pin1 +/-; KO = Pin1 -/-).

Figure 7. Pin1 null MEFs exhibit a defect in global protein synthesis.



Primary MEFs were seeded into 6 well dishes at 400,000 cells/well in the presence of 10% heat-inactivated FBS. The following day, serum was removed and cells were starved for 48 hours. After 48 hours, the cells placed in methionine-free media for 30 minutes. S^{35}-Methionine was then added to the wells either alone, or at the same time as 10% dialyzed FBS. Cells were lysed at various times after the addition of S^{35}-Methionine, and the lysates were quantified, TCA precipitated, and analyzed using a scintillation counter. S^{35}-Methionine incorporation in serum stimulated cells was normalized against the incorporation that occured in unstimulated (serum starved) cells by subtracting the CPM values of serum starved cells from the CPM values of serum stimulated cells at the corresponding timepoints after S^{35}-Methionine addition. This is one representative of three experiments showing a defect in S^{35}-Methionine incorporation in Pin1 null MEFs.

Figure 8. Pin1 null mice exhibit a defect in producing IL-6 in response to LPS.



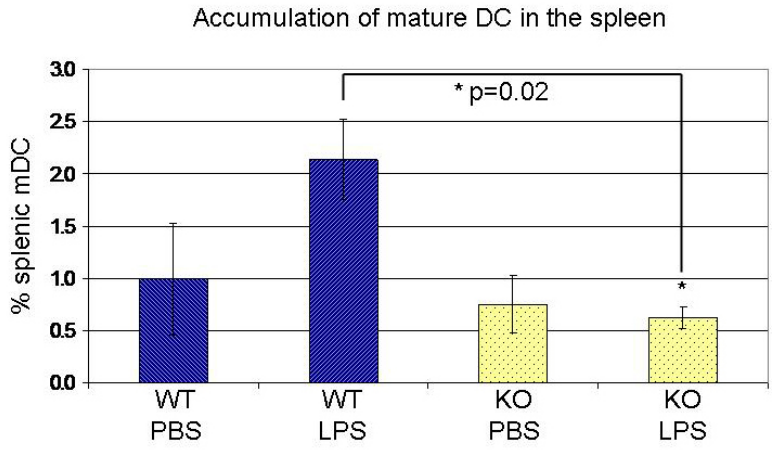
WT and Pin1 null mice (KO) were administered PBS alone or 15-25mg/kg LPS by I.P. injection. Seventeen hours later, mice were sacrificed. Serum was collected and analyzed by ELISA for IL-6 production.

Table 1. Serum analysis in WT and Pin1 null mice by Rules-Based Medicine, Inc.

SERUM FACTOR	UNIT	WT PBS	KO PBS	WT LPS	KO LPS
GM-CSF	pg/ml	ND	ND	96	9.2
IFNγ	pg/ml	ND	ND	1670	90
IL-1α	pg/ml	521	303	1910	612
IL-1β	ng/ml	8.1	8.9	15	13
IL-2	pg/ml	9.3	ND	580	141
IL-3	pg/ml	ND	ND	136	21
IL-4	pg/ml	16	16	354	117
IL-5	ng/ml	0.74	1.1	3.7	2.2
IL-6	pg/ml	ND	4.2	>17542	4030
IL-7	ng/ml	0.055	0.099	1.1	0.52
IL-10	pg/ml	199	219	8470	2030
IL-11	pg/ml	ND	19	563	297
IL-12	ng/ml	ND	ND	3.9	1.1
IL-17	ng/ml	ND	ND	3.0	0.17
IL-18	ng/ml	4.7	4.9	40	10
LIF	pg/ml	701	669	26800	2610
MIP-1α	ng/ml	0.78	0.85	1.6	1.1
MIP-1β	pg/ml	82	88	13700	1140
MIP-2	pg/ml	24	21	>7372	750
RANTES	pg/ml	0.88	0.52	243	43
TNFα	ng/ml	0.034	ND	1.7	0.72
VEGF	pg/ml	192	164	2310	1090

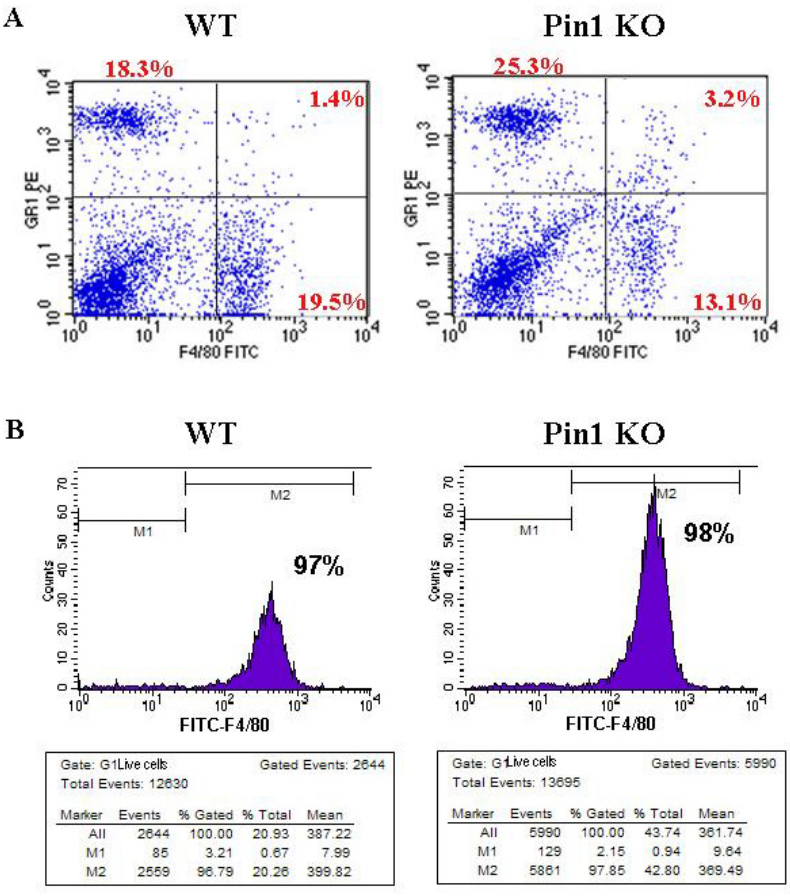
Seventeen hours after I.P. administration of either LPS or PBS, mice were sacrificed. Serum was pooled for 3 WT mice + LPS (WT LPS), 2 WT mice + PBS (WT PBS), 3 Pin1 KO mice + LPS (KO LPS), and 2 Pin1 KO mice + PBS (KO PBS). It was then sent to RBM, Inc for analysis. ND = Not Detected; (>) = above limit of detection.

Figure 9. Pin1 null mice fail to accumulate splenic mDC in response to LPS.



PBS or LPS was administered to mice by I.P. injection. Seventeen hours later, mice were sacrificed. Splenocytes were isolated and stained for markers of mature dendritic cells (mDC). Cells were then analyzed by flow cytometry.

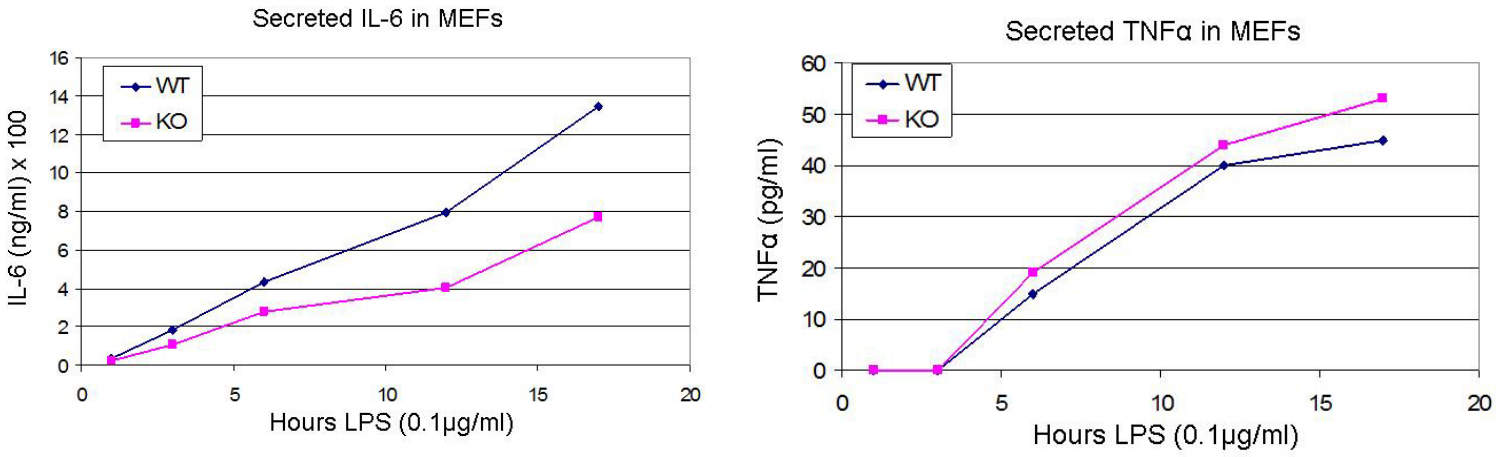
Figure 10. Macrophages and neutrophils exist in the peritoneal cavity of Pin1 null mice.



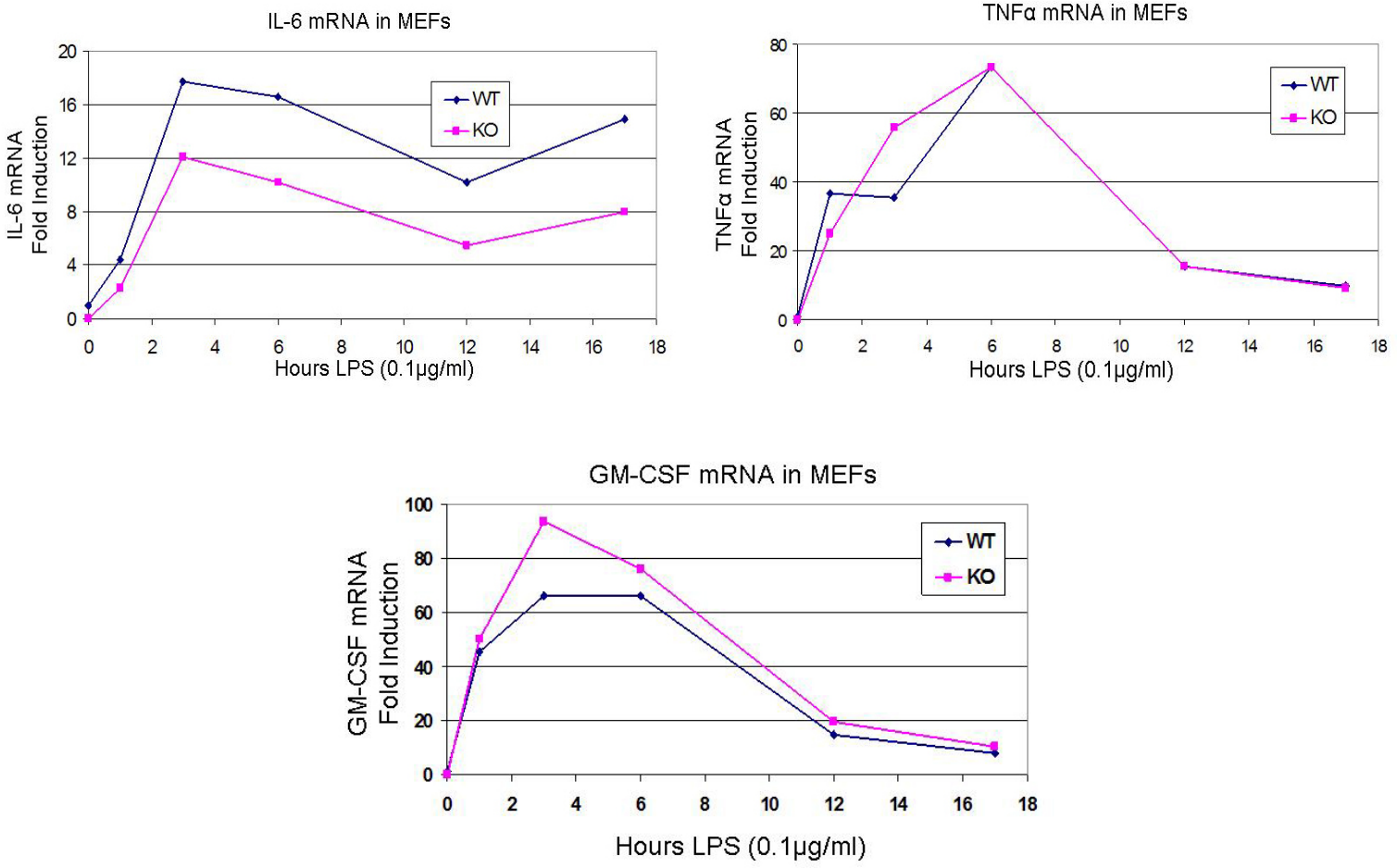
A) Cells obtained from the peritoneal cavity of healthy mice were stained for macrophage and neutrophil markers (CD11b, GR1, F4/80). All plotted cells are CD11b+. Upper Left Quadrant (F4/80-GR1+) represents Neutrophils; Upper Right + Lower Right Quadrants (All F4/80+) represent Macrophage. **B)** Cells from peritoneal flush were placed in culture overnight. Adherent cells were removed and stained with the macrophage marker F4/80. Increased counts in Pin1 KO are due to the increase in gated events, indicated in the chart below.

Figure 11. Primary Pin1 null MEFs are defective in IL-6 mRNA accumulation and protein secretion.

A



B



Primary MEFs were seeded at 400,000 cells/well into a 6 well plate and then left overnight (14 hrs). The next day, LPS was added to the media for various amounts of time. Media was collected at each time point and analyzed by ELISA **(A)**, and total RNA was isolated and analyzed by q-rtPCR **(B)**.